lanl2dz basis set developed by Hay and Wadt (this basis set employs an effective core potential on zinc). Both basis sets were used as implemented in the standard basis set library of Gaussian98. The lanl2dz basis set not only gave structural data that were directly comparable to the larger all-electron basis, but also eliminated self-consistent-field (SCF) convergence problems which occurred when the 6-311 + G(d,p) basis set was used for the larger systems. Atomic charges and hyperconjugative interaction energies were obtained by using the natural bond orbital analysis of Reed et al.[18] as implemented in Gaussian98.

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Received: July 16, 2001 [Z266]

Breakdown of Chlorophyll: Electrochemical Bilin Reduction Provides Synthetic Access to Fluorescent Chlorophyll Catabolites

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KEYWORDS:

catabolite · chlorophyll · electrochemistry · enzyme catalysis · tetrapyrrole

Chlorophyll catabolites from plants have long remained undiscovered, and chlorophyll breakdown was elusive until about ten years ago.[1, 2] In contrast to all earlier expectations, the degradation of chlorophylls in senescent vascular plants rapidly progresses to colorless chlorophyll catabolites.[1, 3] Trace amounts of fluorescent compounds were detectable as intermediates in the breakdown of chlorophyll to the colorless catabolites.[4] Minute samples of the two fluorescent chlorophyll catabolites (FCCs) **2a**[5] and **2b**[6] could be prepared with active enzyme extracts from pheophorbide a (**1**). The FCCs **2a** and **2b** are epimeric at their C(1) centers[7] and are identified as the "primary" FCCs (pFCCs) of the two known (stereodivergent) paths of chlorophyll catabolism in higher plants (Scheme 1).[8]

Enzymatic conversion of **1** to pFCCs requires pheophorbide a oxygenase (PaO), which oxygenates **1** to the elusive red chlorophyll catabolite (**3**, RCC), and RCC reductase (RCCR), which acts jointly with PaO to reduce RCC to one of the two epimeric pFCCs.[3, 9, 10] Two types of RCCRs have thus evolved in higher plants[8] which have no cofactor, but use reduced ferredoxin.[10] The enzymatic reduction of RCC may therefore occur by one-electron steps.

We have set out to find an efficient preparative route to pFCCs by means of a non-enzymatic synthesis, and to specifically examine, for this purpose, the capacity of an electrochemical reduction of RCC (**3**).[11] Herein we report on electro-synthetic studies, which have resulted in a preparative route to both epimeric lines of fluorescent chlorophyll catabolites. For practical reasons (stability problems encountered with the pFCC's **2a**/**2b**) the work was carried out with RCC methyl ester **4**.[11] The reduction of **4** to the methyl esters of the two epimeric pFCCs (**5a** and **5b**; Scheme 2) was achieved by electrolysis in a two compartment cell: a deoxygenated solution of **4** in MeOH was electrochemically reduced at an Hg electrode at -1.3 V versus a 0.1 normal calomel electrode (NCE) reference.[12] The reaction mixture was analyzed and purified by HPLC (see Figure 1 and Scheme 2). Four homogeneous fractions (**5a'** (ca. 1%), **5a** (12%),

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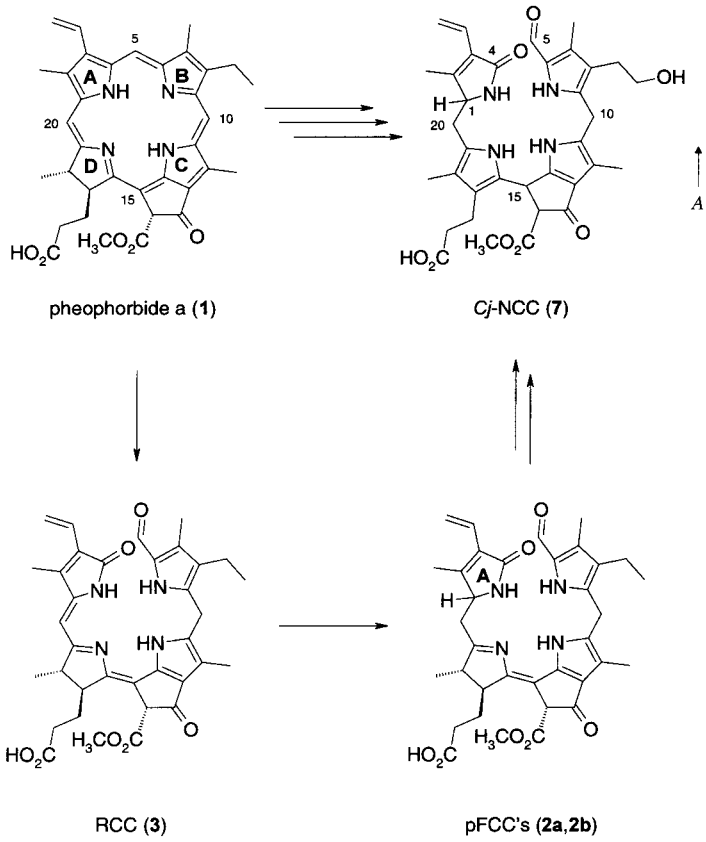
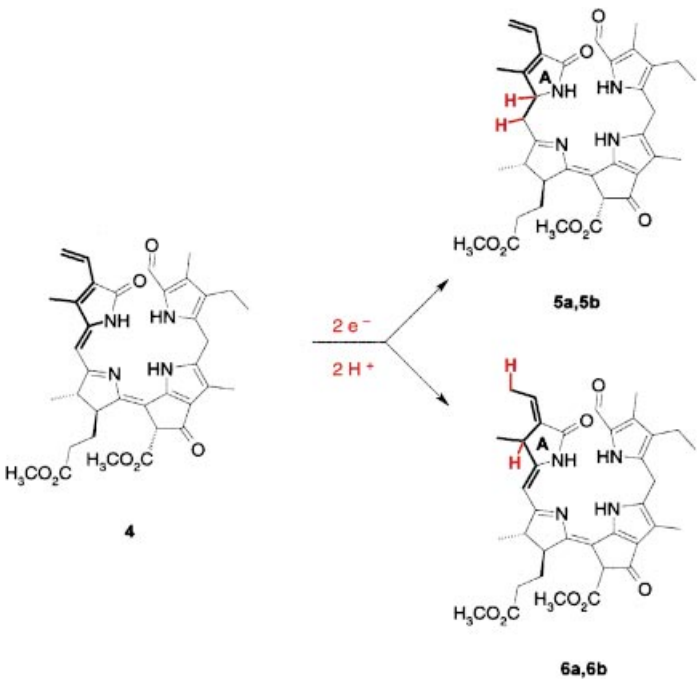


Scheme 1.



Scheme 2.

5b (9%), and **5b'** (4.5%)) with UV/Vis spectra characteristic of FCCs were obtained. Two slightly less polar major fractions (**6a** (20%) and **6b** (7%)) contained side products with a common

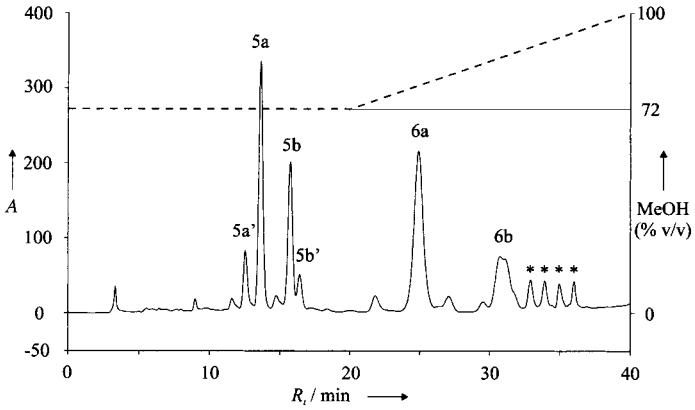


Figure 1. *Electrochemical reduction of **4**. Analysis of the reaction mixture by HPLC (fractions are labeled according to the compound number given in the text; * signifies an unidentified fraction with a similar UV/Vis spectrum as **6a**). Conditions: Hypersil ODS 5 μm; MeOH/water, gradient profile as indicated; detection at 320 nm.*

new chromophore. The molecular formula $C_{36}H_{42}N_4O_7$, consistent with formal H_2 addition to **4**, was derived by mass spectrometric analysis of all six fractions.

The esters **5a** and **5b** were confirmed by spectroscopy as 3^1,3^2-didehydro-13^2-methoxycarbonyl-17^4-methyl-4,5-seco-4,5-dioxo-1,4,5,10,17,18,20-(22H)-octahydrophytoporphyrinates: The UV/Vis spectrum of **5a** in MeOH showed absorption maxima at 317 and 358 nm (Figure 2), similar to that of **5b** and of the pFCCs (**2a** and **2b**).[5, 6] Likewise, the fluorescence spectra of **5a** and **5b** in MeOH exhibited a maximum at about 436 nm, similar

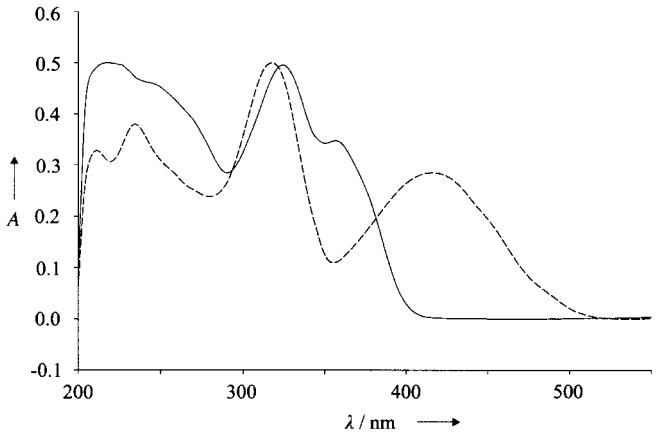


Figure 2. *UV/Vis spectra of fractions **5a** (——) and **6a** (– – –) from on-line diode-array detection (see Figure 1 for the HPLC profile).*

to that of the pFCCs. Analysis of **5a** and **5b** by NMR spectroscopy (Figure 3) provided signal assignments for 31 C atoms, as well as of 38 H atoms, that is, of all of the carbon-bound H atoms except for the exchanging H($C13^2$) atom. The NOE data confirmed the suggested (relative) configurations at $C(13^2)$, C(17), and C(18), and indicated that **5a** and **5b** were C(1) epimers, but the question of the absolute configuration of the C(1) atom in the FCCs is still unanswered.[13] The minor components **5a'** and **5b'** interconverted slowly with **5a** and **5b**,

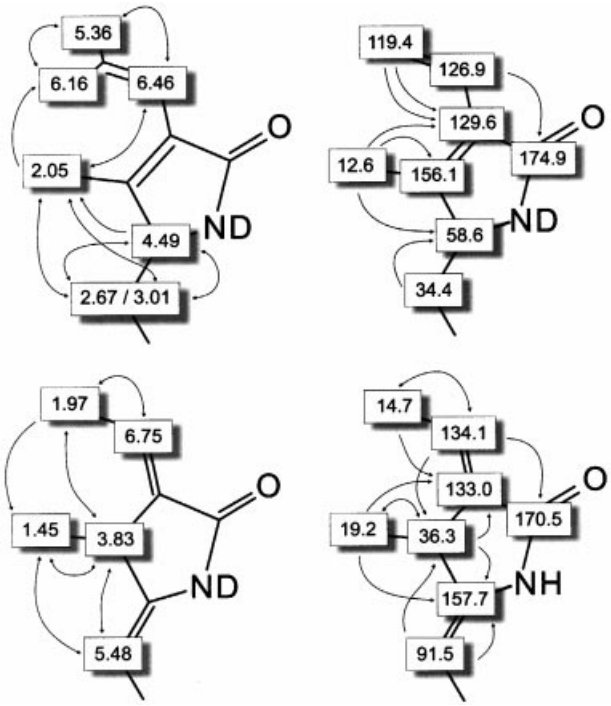


Figure 3. *NMR characterization of rings A of* ***5a*** *(top) and* ***6a*** *(bottom). Left: ^{1}H NMR signal assignments and NOE correlations from ROESY spectra (500 MHz, CD_3OD). Right: ^{13}C NMR signal assignments from ^{1}H,^{13}C-HSQC, and ^{1}H,^{13}C-HMBC spectra of* ***5a*** *(125 MHz, CD_3OD) and* ***6a*** *(75 MHz, $CDCl_3$); the arrows point from the ^{1}H site to the correlated ^{13}C sites.*

respectively, by epimerization at C(13^2) (as determined by HPLC and NMR spectroscopy).

The UV/Vis spectra of the tetrapyrrole fractions **6a** and **6b** showed two prominent absorption maxima at 317 and 416 nm (Figure 2), which indicates they have a common chromophore structure different from that of the FCCs. The NMR spectra of the tetrapyrrole fraction **6a** (Figure 3) enabled assignment of all the 39 carbon-bound H atoms and of 34 C atoms, and showed **6a** to be a 3^1-dehydro-13^2-methoxycarbonyl-17^4-methyl-4,5-seco-4,5-dioxo-2,4,5,10,17, 18,(22H)-heptahydrophytoporphyrinate. A similar NMR analysis indicated **6b** to have the same constitution as **6a**, but to differ in the configuration at C(13^2). Both fractions (**6a** and **6b**) were roughly (1:1) mixtures of two epimers (epimeric at C(2)) with a uniform *E* configuration of the exocyclic double bond C(3)–C(3^1). The electroreduction of **4** to **6a**/**6b** thus leads to a bilin with a β-ethylidene function in an unprecedented and remarkably efficient way.[14]

The stereo-unselective reduction of **4** to the FCC methyl esters **5a** and **5b** and to their isomers **6a** and **6b** proceeds by a sequence of one-electron reduction and protonation steps similar to an electrochemical ecec mechanism.[15] These transformations may involve protonation steps other than those reflected by the product structures: a third chromophore type, with absorption maxima near 320 and 460 nm, can be observed transiently (at partial conversion) by HPLC analysis.

Our studies indicate RCC to have an inherent propensity for reduction to FCCs by one-electron reducing agents, which is consistent with the noted absence of a cofactor in RCCR. These findings suggest the major tasks of the reductase in the enzymatic formation of pFCCs to concern the regio- and stereoselective protonation steps (besides the rapid interception of oxygenase-bound RCC) and recruitment of reduced ferredoxin. In addition, as the half-wave potential for the reduction of **4**[12] is more negative than that of ferredoxins,[16] the protein-induced modulation of the redox properties of the elusive enzyme-bound form of RCC may be another crucial factor in enzymatic pFCC synthesis (this is the subject of further electrochemical studies).

The electroreduction of **4** into two types of isomeric reduction products (such as **5a** and **6a**, see Scheme 2) is reminiscent of the spectrum of ferredoxin-dependent enzymatic biliverdin reductions, which provide phycobilins in plants, cyanobacteria, red algae, and cryptomonads.[17, 18] The reduction of **4** to **5a** and **6a** is specifically reminiscent of the reductions of the heme catabolite biliverdin[17] to the phycobilins 15,16-dihydrobiliverdin or phytochromobilin by ferredoxin-dependent bilin reductases,[18] which indeed show sequence homology to RCCR.[19] These reductases may also share a functional similarity with RCCR: A parallel between chlorophyll breakdown in vascular plants[3] and heme catabolism in photoorganisms[18, 19] becomes apparent.

The reduction of the formylbilin **4** to **5a** and **5b** opens up a non-enzymatic preparative route to "primary" FCC's, which appear fleetingly as the first non-green catabolites of chlorophyll breakdown in higher plants. The availability of **5a** and **5b** enables chemical investigations of the tautomerization of FCCs to nonfluorescent chlorophyll catabolites (NCCs), such as *Cj*-NCC (**7**; Scheme 1). Such studies may help to judge the role of possible enzyme catalysis in this late transformation during chlorophyll breakdown in higher plants.

Experimental Section

A solution of **4** (1.0 mg, 1.56 μmol), $LiClO_4$ (0.1 M), and phenol (0.5 mM) in MeOH (30 mL) in the cathode compartment of an electrolysis cell in a glove box (N_2, <10 ppm O_2) was reduced at -1.3 V versus a 0.1 N calomel reference electrode (0.1 NCE) at an Hg electrode. A solution of **4** (10.4 mg, 16.2 μmol)[11] and phenol (15.0 mg, 160 μmol) in MeOH (2.6 mL, 0.038 M $LiClO_4$) was continuously added slowly over 2 h, so that the current remained constant at 325 ± 25 μA. Analysis by HPLC (see Figure 1) indicated nearly complete conversion after a consumption of 3.76 C (2.2 F mol^{-1}, 150 min). The crude product (diluted with CH_2Cl_2, extracted with 100 mM potassium phosphate at pH 7, and dried) was purified by HPLC (column: Hypersil ODS 5 μm 21.6 × 250 mm; eluent: 0–68 min 62:38 vol% MeOH:H_2O, after 60 min 72:28 vol% MeOH:H_2O; flow: 10 mL min^{-1}; UV detection: 320 nm). Fractions were collected after 40 (**5a'**), 48 (**5a**), 57 (**5b**), 64 min (**5b'**), 85 (**6a**), and 93 min (**6b**). Each fraction was diluted with water (1:1), loaded on a Sep-Pak C_{18} cartridge, washed with water (ca. 20 mL), and eluted with MeOH (3.5 mL). The solvents were evaporated in vacuo at $T < 0\,^\circ$C to give 0.1 mg (0.9%) of **5a'**, 1.3 mg (11.4%) of **5a**, 1.0 mg (8.6%) of **5b**, 0.5 mg (4.4%) of **5b'**, 2.3 mg (20.1%) **6a**, and 0.8 mg (7%) **6b**, overall 52.4%.

Selected spectroscopic data: ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) in CD_3OD, signal assignment from ^{1}H,^{13}C-HSQC, and ^{1}H,^{13}C-HMBC. FAB-MS: positive ion mode, glycerol matrix. **5a**: UV/Vis

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(MeOH): λ_{max} (log ε) = 317.5 (4.37), 358.5 (4.24); ^{1}H NMR: δ = 0.98 (t, J = 7.8 Hz, H_3–C(8^2)), 1.11 (d, J = 6.8 Hz, H_3–C(18^1)), 1.64 and 1.92 (2 × m, H_2–C(17^1)), 2.05 (s, H_3–C(2^1)), 2.12 (s, H_3–C(12^1)), 2.25 (s, H_3–C(7^1)), 2.27 (m, H_2–C(17^2)), 2.41 (q, J = 7.8 Hz, H_2–C(8^1)), 2.43 (m, H–C(17)), 2.66 (m, H–C(18)), 2.67 (m, H_A–C(20)), 3.01 (dd, J = 16.6, 3.9 Hz, H_B–C(20)), 3.60 (s, H_3–C(17^5)), 3.73 (s, H_3–C(13^5)), 4.00 (s, H_2–C(10)), 4.49 (m, H–C(1)), 5.36 (dd, J = 11.7, 2.0 Hz, H_{cis}–C(3^2)), 6.16 (dd, J = 17.6, 2.0 Hz, H_{trans}–C(3^2)), 6.46 (dd, J = 17.6, 11.7 Hz, H–C(3^1)), 9.37 (s, H–C(5)); ^{13}C NMR: δ = 8.8 (C(7^1)), 9.1 (C(12^1)), 12.6 (C(2^1)), 15.3 (C(8^2)), 17.6 (C(8^1)), 18.1 (C(18^1)), 23.5 (C(10)), 28.9 (C(17^1)), 31.6 (C(17^2)), 34.4 (C(20)), 47.7 (C(17)), 51.8 (C(18)), 52.0 (C(17^5)), 52.9 (C(13^5)), 58.6 (C(1)), 112.5 (C(12)), 119.4 (C(3^2)), 126.3 (C(8)), 126.9 (C(3^1)), 127.1 (C(13)), 129.5 (C(6)), 129.6 (C(3)), 133.8 (C(7)), 136.1 (C(11)), 136.4 (C(9)), 156.1 (C(2)), 170.5 (C(13^3)), 174.8 (C(17^3)), 174.9 (C(4)), 177.8 (C(5)), 186.1(C(19)); FAB-MS: *m/z*(%): 644.4(45), 643.4(100, exp. mass for $[M+H]^+$: 643.317 ± 0.005), 521.3(29) $[M+H-C_7H_8NO]^+$, 506.2(15) $[M+H-C_8H_{11}NO]^+$. **5b:** UV/Vis (MeOH): λ_{max} (log ε) = 319 (4.33), 358.5 (4.17); ^{1}H NMR: δ = 0.99 (t, J = 7.8 Hz), 1.11 (d, J = 7.8 Hz), 1.72 and 1.90 (2 × m), 2.07 (s), 2.13 (s), 2.24 (s), 2.27 (m), 2.40 (m), 2.43 (m), 2.61 (m), 2.64 (m), 3.08 (dd, J = 17.6, 3.9 Hz), 3.59 (s), 3.72 (s), 4.01 (s), 4.49 (m), 5.37 (dd, J = 11.7, 2.9 Hz), 6.19 (dd, J = 17.6, 2.9 Hz), 6.51 (dd, J = 17.6, 11.7 Hz), 9.36 (s); ^{13}C NMR: δ = 8.7, 8.9, 12.2, 15.2, 17.4, 17.9, 23.1, 28.7, 31.4, 33.9, 47.4, 51.8 (C(17^5)), 52.7 (C(18)), 52.7 (C(13^5)), 58.2, 112.4, 119.2, 126.2, 126.6, 127.0, 129.7, 129.7, 133.9, 136.2, 136.7, 155.8, 170.6, 174.9, 177.6, 186.0; FAB-MS: *m/z*(%): 643.4(100, exp. mass for $[M+H]^+$: 643.320 ± 0.007). **6a**: UV/Vis (MeOH:H_2O (4:1)): λ_{max} (rel ε) = 317 (1.00), 416 (0.57); ^{1}H NMR: δ = 0.99 and 1.00 (2 × t, J = 7.8 Hz, H_3–C(8^2)), 1.12 and 1.13 (2 × d, J = 7.8 Hz, H_3–C(18^1)), 1.44 and 1.45 (2 × d, J = 7.8 Hz, H_3–C(2^1)), 1.71 and 1.94 (m, H_2–C(17^1)), 1.97 (d, J = 7.8 Hz, H_3–C(3^2)), 2.03 (s, H_3–C(12^1)), 2.27 (s, H_3–C(7^1)), 2.32 (m, H_2–C(17^2)), 2.39 (m, H–C(17)), 2.44 and 2.45 (2 × q, J = 7.8 Hz, H_2–C(8^1)), 2.72 (m, H–C(18)), 3.62 and 3.63 (2 × s, H_3–C(17^5)), 3.73 (s, H_3–C(13^5)), 3.83 (m, H–C(2)), 4.05 (s, H_2–C(10)), 4.45 (s, H–C(13^2)), 5.48 (s, H–C(20)), 6.75 (m, H–C(3^1)), 9.39 (s, H–C(5)); ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ = 8.8 (C(7^1)), 9.2 (C(12^1)), 14.5 (C(8^2)), 14.7 (C(3^2)), 16.6 (C(8^1)), 18.9 (C(18^1)), 19.2 (C(2^1)), 22.8 (C(10)), 27.8 (C(17^1)), 30.9 (C(17^2)), 36.3 (C(2)), 46.1 (C(17)), 49.2 (C(18)), 51.5 (C(17^5)), 52.3 (C(13^5)), 60.5 (C(13^2)), 91.5 (C(20)), 111.6 (C(12)), 111.7 (C(15)), 124.9 (C(8)), 126.8 (C(13)), 128.7 (C(6)), 131.0 (C(9)), 131.3 (C(7)), 133.0 (C(3)), 133.8 (C(11)), 134.1 (C(3^1)), 154.1 (C(16) or C(14)), 157.7 (C(1)), 168.4 (C(13^3)), 170.5 (C(4)), 172.7 (C(17^3)), 176.6 (C(5)), 179.8 (C(19)), 185.9 (C(13^1)); FAB-MS: *m/z*(%): 644.4(55), 643.4(100, exp. mass for $[M+H]^+$: 643.312 ± 0.005), 507.2(10, $[M+H-C_8H_{11}NO]^+$). **6b**: UV/Vis (MeOH:H_2O (4:1)): λ_{max} (rel. ε) = 317 (1.00), 416 (0.57); ^{1}H NMR: δ = 0.97 (m), 1.12 and 1.13 (2 × d, J = 6.8 Hz), 1.43 and 1.45 (2 × d, J = 7.8 Hz), 1.50 and 1.69 (m), 1.99 (d), 2.03 and 2.04 (2 × s), 2.27 (s), 2.30 (m), 2.43 (m), 2.61 (m), 2.69 (m), 3.64 and 3.65 (2 × s), 3.72 (s), 3.83 (m), 4.04 (s), 4.32/4.33 (d), 5.47 (s), 6.77 (m), 9.39 (s); FAB-MS: *m/z*(%): 643.4(100, exp. mass for $[M+H]^+$: 643.317 ± 0.004).

We would like to thank Christian Eichmüller and Robert Konrat for their help with NMR spectra, and Kathrin Breuker and Karl-Hans Ongania for measuring FAB mass spectra. We are grateful to the Austrian National Science Foundation (FWF, project P-13503) for financial support.

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[13] So far, spectroscopic data and chemical correlation (tautomerization of **5a** and **5b** to the corresponding NCCs, M. Oberhuber, B. Kräutler, unpublished results) provide a tentative correlation of **5a** with **2a**, and **5b** with **2b.**

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Received: August 14, 2001 [Z285]